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Short research
paper

Received: 2004.08.15

Accepted: 2004.10.15

Published: 2005.01.05

Partial mitochondrial genome sequences of *Ostrinia nubilalis* and *Ostrinia furnicalis***Brad S. Coates**^{1,2}, **Douglas V. Sumerford**¹, **Richard L. Hellmich**^{1,3}, **Leslie C. Lewis**^{1,3}¹USDA-ARS, Corn Insect and Crop Genetics Research Unit, Genetics Laboratory, c/o Insectary, Iowa State University, Ames, Iowa, 50011²Interdepartmental Genetics Program, Iowa State University, Ames, IA, 50011³Department of Entomology, Iowa State University, Ames, IA, 50011**Abstract**

Contiguous 14,535 and 14,536 nt near complete mitochondrial genome sequences respectively were obtained for *Ostrinia nubilalis* and *Ostrinia furnicalis*. Mitochondrial gene order was identical to that observed from *Bombyx*. Sequences comparatively showed 186 substitutions (1.3% sequence divergence), 170 CDS substitutions (131 at 3rd codon positions), and an excess of transition mutation likely resulting by purifying selection ($d_N/d_S = \omega \cong 0.15$). Overall substitution rates were significantly higher at 4-fold (5.2%) compared to 2-fold degenerate codons (2.6%). These are the 3rd and 4th lepidopteran mitochondrial genome reference sequences in GenBank and useful for comparative mitochondrial studies.

Key words*Ostrinia nubilalis*, *Ostrinia furnicalis*, mitochondrial variation**Author
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1. Introduction

Mitochondrial genomes of 16 insect species are completely sequenced and published with a majority from the order Diptera; *D. yakuba* [1], *Ades gambiae* [2], *Anopheles quadrimaculatus* [3], *D. melanogaster* [4], *Ceratitis capitata* [5], *Cochliomyia hominivorax* [6], *D. simulans* [7], and *Bactrocera oleae* [8]. Complete sequences also have been published from a hymenopteran, *Apis mellifera* [9], an orthopteran, *Locusta migratoria* [10], a phthirapteran *H. macropus* [11], thysanuran, *T. inaginis* [12], a hemipteran *Triatoma dimidiata* [13], coleopteran *Crioceris duodecipunctata* [14], and lepidopterans *Bombyx mori* and *B. mandarina* [15].

Larvae from corn borer species *Ostrinia nubilalis* and *Ostrinia furnicalis* (Lepidoptera: Crambidae) are pests of agricultural crop plants and cause major crop production losses [16, 17]. *Ostrinia nubilalis* and *O. furnicalis* are sister species [18, 19], with difference residing in female *O. nubilalis* and *O. furnicalis* emission of E- and Z-stereoisomers of $\Delta 11$ - [20], and $\Delta 12$ -tetradecenyl acetates [21], respectively. The pheromone binding protein gene sequences showed little nucleotide variance between *O. nubilalis* and *O. furnicalis* [22], and 7 allozyme markers indicated a high similarity between Chinese populations of *O. nubilalis* and *O. furnicalis* suggesting recent speciation [18]. Similarly, mitochondrial cytochrome c oxidase subunit II (*coxII*) gene alignment estimated 1.63% interspecies divergence [19]. The present study compares GenBank annotated mitochondrial genomes from *O. nubilalis* (accession AF442957) and *O. furnicalis* (AF467260).

2. Materials and methods

2.1 Samples and amplification

A single bivoltine female Z-pheromone race *O. nubilalis* adult was collected from the Iowa State University Uthe Farm, Ames, Iowa, USA. One adult multivoltine *O. furnicalis* female of indeterminate pheromone composition collected from Hengshui, Hebei Province, China was contributed by Dr. Wang Zhen-ying, Institute of Plant Protection, Chinese Academy of Agricultural Sciences, Beijing, China. DNA extractions used Qiagen DNeasy kits (Qiagen, Valencia, CA).

Primers combinations TY-J-1460 with TK-N-3785, J-11545 with N-12854, and N1-J-12585 with SR-N-14588 [23] were used to PCR amplify fragments 2, 9, and 10 (Fig. 1). *Bombyx mori* (GenBank:AF149768 and AY048187) and *D. yakuba* (GenBank: MIDYRRN) [1] mitochondrial genomes were aligned using AlignX software (Informax, San Francisco, CA) to identify regions of sequence similarity, from which regions PCR primers were designed to amplify remaining fragments using Primer3 [24]. All PCR reactions were performed in a 50 μ l volume with 1.7 U of *Tli* polymerase (Promega Corp., Madison, WI), 100 ng of DNA, 5 μ l 10X thermal polymerase buffer (Promega), 2.5 mM MgCl₂, 200 μ M dNTPs, and 20 pmol of each primer. Fragments 1, and 3 to 8 were amplified on a PTC-100 thermocycler (MJ Research, Watertown, MA) with denaturation at 95°C for 2 m, followed by 40 cycles at 94°C for 30 s, 50 to 54 °C for 40 s, a 2.5 °C/s ramp for +15 °C, and 70 °C for 1.5 to 3 m depending on fragment length. Fragments 2, 9, and 10 were amplified by denaturing template at 95°C for 2 m, followed by 40 cycles at 94°C for 30 s, 44 °C for 1 m, a 2.0 °C/s ramp for +23 °C, and 70 °C for 3 m.

2.2 DNA sequence and analysis

PCR reaction products for fragments 1 to 10 were purified using Qiaquick PCR purification columns (Qiagen), and diluted to 2.5ng/ μ l/100 bp of product length. Sequencing was performed in duplicate at the DNA sequencing core facility at Iowa State University, Ames, IA. Overlapping fragments were assembled into a single contiguous sequence using Contig Express software (Informax). *Ostrinia nubilalis* and *O. furnicalis* mitochondrial genome sequences were aligned with *B. mori* (GenBank AF149768, and GenBank AY048187) using AlignX software (Informax), and gene features were annotated using Vector NTI 7.0 (Informax). Contiguous mitochondrial DNA sequence of 14535 and 14536 nt were respectively submitted to GenBank for *O. nubilalis* (AF442957) and *O. furnicalis* (AF467260).

Substitution rate and transition/transversion ratio for *Ostrinia* mitochondrial DNA sequences were calculated with MacClade 4.03 [25]. Twenty one tRNA gene structures were predicted with M-fold 3.1 [26], and viewed using RNAviz 2.0 [27]. Codon usage was evaluated by the Countcodon program version 4 (<http://www.kazusa.or.jp/codon/countcodon.html>). Average per site rates of synonymous (d_S) and nonsynonymous nucleotide substitution (d_N) were calculated according to [28] using MEGA [29].

3. Results and discussion

3.1 *Ostrinia* mitochondrial genomes

Contiguous *O. nubilalis* (GenBank accession: AF442957) and *O. furnicalis* (AF467260) mitochondrial genomes were assembled from overlapping PCR product sequence (Fig. 1). Each GenBank record includes 13 open reading frames (ORFs), a large ribosomal RNA (*rrnL*) gene, 21 tRNAs, and part of *trnM* and small ribosomal RNA (*rrnS*) genes (Fig. 1). Gene order and orientation were identical to *Drosophila* [1, 4], except for translocation of *trnM* to a position preceding *trnI* as was observed in *Bombyx* [15]. Major strand of *O. nubilalis* (41.3% A, 38.8% T, 8.0% G, and 11.8% C; 80.2% AT) and *O. furnicalis* (41.5% A, 38.9% T, 7.9% G, and 11.7% C; 80.4% AT) showed a bias toward A and T nucleotides that is typical of insect mitochondrial genomes [30].

The *O. nubilalis* and *O. furnicalis* mitochondrial genomes have 3731 codons; 3718 amino acid encoding and 13 termination codons (Table 1). Codons had a prevalence of A and T in 3rd positions and bias may reflect selection for optimal tRNA use [31], speed of genome replication, genome bias, or DNA repair efficacy (Table 2).

The *O. nubilalis* and *O. furnicalis* mitochondrial peptides comparatively showed 24 predicted amino acid changes (24 of 3718; 0.646%; peptide similarity \cong 99.22%, and identity \cong 99.78%) [32]. All ORFs were initiated by ATA or ATT codons, except *cox1*. Initiation of *cox1* translation is ambiguous, but may occur by a TATTAG sequence in *O. nubilalis* and *O. furnicalis*, that is similar to TTTTAG in the *B. mori*. Hexanucleotides, initiation signals TATCTA from *Penaeus monodon* [33], or ATTTAA from *A. gambiae* [2], *A. quadrimaculatus* [3] and *C. capitata* [5] have been proposed. Alternatively, an ATAA tetranucleotide sequence was predicted to initiate *cox1* translation in *Drosophila*, *L. migratoria* [10], and *Daphnia pulex* [34]. Termination codons were either TAA or TAG in *O. nubilalis* and *O. furnicalis*, except for *cox2* and *atp6* that have incomplete stop codons T and TA, respectively. Incomplete stop codons may become function after polycistronic transcript cleavage and polyadenylation mechanisms [35, 36].

Complete nucleotide sequence was obtained for 21 *O. nubilalis* and *O. furnicalis* mitochondrial tRNAs. Seven substitutions were observed, and 0.49% sequence divergence was estimated from 1429-shared sites. Insertion-deletion (indel) mutation occurred in loop structures of *trnA*, *trnD*, *trnG*, and *trnT*, and, except for *trnR*, did not affect predicted two-dimensional tRNA structures (Fig. 2). Variable mitochondrial tRNA loops in *Bombyx* were assumed not to affect biological function [15]. The complete *rrnL* gene sequence was 1339 nt for *O. nubilalis* and *O. furnicalis*, and alignment comparatively showed a single C to T transition. A partial *rrnS* sequence was obtained from *O. nubilalis* (434 nt) and *O. furnicalis* (435 nt), and comparatively showed a single nucleotide deletion.

3.2 Nucleotide substitution pattern

A 14543 nt consensus mitochondrial genome alignment identified 186 substitutions between *O. nubilalis* and *O. furnicalis*: 138 transition (ts) and 48 transversion (tv) mutations (ts:tv = $\kappa \cong$ 2.88). This ratio deviated significantly from neutral expectation (1:2; $\chi^2 = 141.447$, d.f. = 1, $P < 0.001$), indicating evolutionary pressures are acting upon *O. nubilalis* and *O. furnicalis* mitochondrial genomes. Excess transition mutation also was reported between *D. melanogaster* subgroup members ($\kappa = 761/180 \cong 4.23$) and attributed to non-neutral evolutionary forces or population effects [7].

Additionally, mitochondrial protein coding sequences (CDS) comparatively showed 170 substitutions between *O. nubilalis* and *O. furnicalis*; 131 at 3rd codon positions. The ratio of the rate of nonsynonymous changes at nonsynonymous sites (d_N) to synonymous changes at synonymous sites (d_S) in *Ostrinia* ORFs indicated a 7-fold excess of silent mutation ($d_N/d_S = \omega \cong 0.15$) [28]. High peptide similarity (\cong 99.22%) may reflect regency *O. nubilalis* and *O. furnicalis* speciation, but effects of purifying selection can be inferred since synonymous substitutions are very prevalent. Alternatively, similar environmental selection after speciation could lead to peptide conservation co-occurring with a background of random genetic drift at neutral nucleotide positions. The observed mutation rate at *Ostrinia* 4-fold degenerate codons ($\mu_{4\text{-fold}} = 5.22\%$) was significantly higher than at 2-fold degenerate codons ($\mu_{2\text{-fold}} = 2.60\%$; $\chi^2 = 35.157$, d.f. = 1, $P < 0.001$). Results suggest a greater susceptibility of 4-fold degenerate codons to synonymous substitution.

3.3 Divergence time estimates

The divergence time between *O. nubilalis* and *O. furnicalis* mitochondrial was estimated by assuming a linear rate of substitution in short-term evolution (molecular clock) [37] of 2% per million years [38]. Nucleotides in rRNA and tRNA may lack independence due to structural dependence, and purifying selection may act at 1st and 2nd codon positions. The intergenic sequence (IGS) and 3rd codon positions only sites that are nearly neutral. IGS region and 3rd codon positions showed 3.54% nucleotide difference between *O. nubilalis* and *O. furnicalis*, indicating that speciation occurred 1.8 mya [38]. Alternatively, 3rd position and IGS region data give a pairwise genetic distance of 0.3284 ± 0.0348 using the Kimura-2-parameter model [39]. Estimates of 0.1 distance unit (D) per 1.0 myr [40] suggest divergence at 3.3 mya. These molecular-based divergence time estimates are supported by highly similar morphology of *O. nubilalis* and *O. furnicalis* [41].

Conflict of interest

The authors have declared that no conflict of interest exists.

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Tables and Figures

Table 1: Codon usage for 3718 amino acid residues and 13 nonsense codons among protein coding regions from each *O. nubilalis* (On) and *O. furnicalis* (Of) using the invertebrate mitochondrial genetic code.

Codon	On	Of	Codon	On	Of	Codon	On	Of	Codon	On	Of
UUU-Phe	347	354	UCU-Ser	93	95	UAU-Tyr	175	170	UGU-Cys	29	30
UUC-Phe	31	28	UCC-Ser	12	10	UAC-Tyr	12	16	UGC-Cys	2	1
UUA-Leu	459	454	UCA-Ser	94	94	UAA-Ter	*12	*12	UGA-Trp	89	89
UUG-Leu	15	17	UCG-Ser	5	4	UAG-Ter	1	1	UGG-Trp	5	5
CUU-Leu	20	22	CCU-Pro	60	60	CAU-His	55	58	CGU-Arg	14	14
CUC-Leu	0	1	CCC-Pro	8	7	CAC-His	7	4	CGC-Arg	1	1
CUA-Leu	32	32	CCA-Pro	55	52	CAA-Gln	61	61	CGA-Arg	33	33
CUG-Leu	0	0	CCG-Pro	1	2	CAG-Gln	3	3	CGG-Arg	3	3
AUU-Ile	449	455	ACU-Thr	71	72	AAU-Asn	232	234	AGU-Ser	23	25
AUC-Ile	28	28	ACC-Thr	14	10	AAC-Asn	24	22	AGC-Ser	2	2
AUA-Met	262	265	ACA-Thr	71	75	AAA-Lys	92	94	AGA-Ser	90	89
AUG-Met	26	20	ACG-Thr	1	0	AAG-Lys	9	8	AGG-Ser	0	1
GUU-Val	72	70	GCU-Ala	70	71	GAU-Asp	59	59	GGU-Gly	56	56
GUC-Val	1	2	GCC-Ala	9	8	GAC-Asp	4	4	GGC-Gly	1	5
GUA-Val	58	62	GCA-Ala	46	47	GAA-Glu	65	62	GGA-Gly	117	115
GUG-Val	6	1	GCG-Ala	2	1	GAG-Glu	8	10	GGG-Gly	29	25

* Includes stop codons from *cox2* (T) and *atp6* (TA), completed by adenylation.

Table 2: Nucleotide frequencies partitioned among *O. nubilalis* (On) and *O. furnicalis* (Of) mitochondrial genome regions. IGS = non-coding intergenic spacer regions.

% nt	Protein Coding Sequence												IGS	
	<u>1st position</u>		<u>2nd position</u>		<u>3rd position</u>		<i>rrnL</i>		<i>rrnS</i>		tRNAs			
	On	Of	On	Of	On	Of	On	Of	On	Of	On	Of	On	Of
% AT	74.4	74.5	70.4	70.5	92.8	93.3	84.9	85.0	82.3	82.8	82.2	82.1	91.9	93.4
% GC	25.6	25.5	29.6	29.5	7.2	6.7	15.1	15.0	17.7	17.2	17.8	17.9	8.1	7.6

Figure 1. *Ostrinia* mitochondrial genome map of sequenced regions. Protein coding genes represented by arrows indicating direction with left-facing arrows on major strand. The tRNA genes are labeled by single letter codes and * indicating coding sequence on minor strand. Underscores indicate positions of ten overlapping PCR amplified genome fragments.

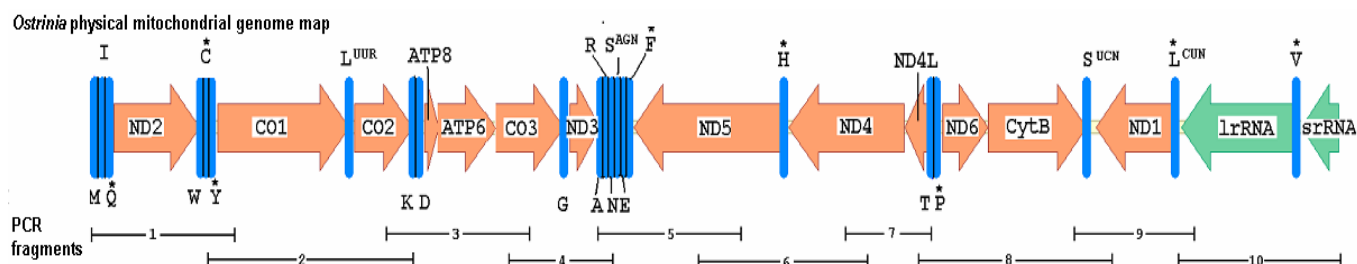


Figure 2: Predicted secondary structures for **A)** *O. nubilalis* and **B)** *O. furnicalis* *trnR*.

